(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 March 2003 (13.03.2003)

PCT

(10) International Publication Number
WO 03/020754 A2

(51) International Patent Classification[7]: C07K 14/00

(21) International Application Number: PCT/GB02/03953

(22) International Filing Date: 29 August 2002 (29.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0120943.6 29 August 2001 (29.08.2001) GB

(71) Applicant *(for all designated States except US)*: UNIVERSITY COURT OF THE UNIVERSITY OF DUNDEE [GB/GB]; Nethergate, Perth Road, Dundee DD1 4HN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PERKINS, Neil, Donald [GB/GB]; Division of Gene Regulation and Expression, School of Life Sciences, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH (GB). ANDERSON, Lisa, Ann [GB/GB]; Division of Gene Regulation and Expression, School of Life Sciences, University of Dundee, MSI/WTB Complex, Dow Street, Dundee DD1 5EH (GB).

(74) Agents: MacDOUGALL, Donald, Carmichael et al.; Cruikshank & Fairweather, 19 Royal Exchange Square, Glasgow G1 3AE (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/020754 A2

(54) Title: INHIBITION OF REPLICATION FACTOR C

(57) Abstract: The present invention relates to the use and provision of an inhibitor of the large subunit of replication factor C [RFC (p140)], and particularly an inhibitor of its interaction with the RelA (p65) NF-kappa B subunit, for the treatment of various medical conditions by inducing apoptosis of cells involved therein.

INHIBITION OF REPLICATION FACTOR C

The present invention relates to the use and provision of an inhibitor of the large subunit of replication factor C [RFC (p140)], and particularly an inhibitor of its interaction with the RelA (p65) NF-kappa B subunit, for the treatment of various medical conditions by inducing apoptosis of cells involved therein.

NF-κB is an important regulator of inflammation, proliferation and apoptosis and its activation integrates a large number of cellular stimuli with changes in gene expression [1,2]. Activation of NF-κB homo- or heterodimers requires their translocation from the cytoplasm to the nucleus and occurs in response to a large number of diverse stimuli [2]. NF-κB subunits have many functions, which can be determined by the circumstances of their activation [1,3]. Prominent amongst these is the ability of the RelA subunit to function as an important regulator of proliferation and apoptosis [4]. RelA can either induce or inhibit these processes dependent on the context in which it is found. For example, RelA containing complexes have been found to be anti-apoptotic in response to tumour necrosis factor alpha (TNF) stimulation and DNA damage induced by ionising radiation and chemotherapeutic agents [1,4]. In contrast, NF-κB has been described as being pro-apoptotic following DNA damage by ultraviolet (UV) light and

activation of the tumour suppressor p53 [1]. The present inventors have been investigating the cellular factors that can determine this decision making process. The specificity with which RelA stimulates gene expression is dependent upon its interactions with transcriptional coactivators and other DNA-binding proteins [1,3]. The present inventors have investigated the ability of the RFC (p140) subunit to function as a regulator of RelA. RFC was originally described as a pentameric complex, which, during the process of DNA replication and repair, facilitates the addition and removal of PCNA [5]. Recent results, however, have suggested more dynamic and diverse cellular functions for RFC (p140). It has been observed in a large complex, termed BASC, which contains the breast cancer susceptibility gene BRCA1 and components of the DNA-repair machinery [6]. RFC (p140) also contains an LXCXE motif through which it binds the retinoblastoma tumour suppressor protein (Rb) and has a pro-survival function following UV stimulation [7]. Rb and BRCA1 are important regulators of transcription and interact directly with DNA-binding proteins such as E2F and p53 respectively [8-10]. The present inventors were interested, therefore, in whether RFC (p140) might also regulate the activity of cellular transcription factors that control cellular proliferation and apoptosis.

Generally, the invention concerns inhibition of the function of the large subunit of replication factor C, RFC (p140). Such inhibition results in the repression of the transcriptional activity of the RelA(p65)NF-kappa B subunit. It also results in RelA induced apoptosis, suggesting that the inhibition of RelA transcriptional activity might be limited to genes associated with preventing apoptosis. This has the effect of converting RelA (and therefore NF-kappaB) from being a transcription factor that is anti-apoptotic to one that stimulates cell death.

It is known that inhibitors of the NF-kappa B pathway have the potential to treat cancer and inflammatory disorders. However, inhibitors that affect the complete pathway may potentially have adverse side effects. The invention describes for the first time that the interaction between RFC and NF-kappaB via RelA is required to prevent cell death from occurring.

In particular, the present invention provides an inhibitor of RFC (p140) activity (and particularly of the interaction between RFC (p140) and RelA) for use in the treatment of a medical condition by the inducement of apoptosis of cells involved in the medical condition.

A corresponding method of treating a mammal is also provided.

The inhibitor may be an inhibitory fragment of RFC (p140), particularly RFC (F1) or RFC (F3); or derivates thereof, such as smaller inhibitory fragments of F1 or F3. Alternative inhibitory agents include antisense constructs comprising nucleotide sequences antisense to the RFC (p140) coding sequence or RFC (F1) or (F3) sequences. Additionally siRNA nucleic acid corresponding to a portion of the RFC (p140) mRNA sequence or RelA mRNA sequence. Examples of such siRNA sequences (sense strand only) are disclosed hereinafter.

These inhibitors may work through disrupting the RelA/RFC(p140) interaction or by inhibiting the functionality of the RelA.RFC(p140) complex.

The invention also provides a pharmaceutical composition which comprises an inhibitor of RFC (p140) and a pharmaceutically acceptable carrier. Suitable carriers are known in the art.

The invention further provides a method of screening for an agent for inducing apoptosis, which comprises assessing a compound for its ability to inhibit RFC (p140).

In vitro and in vivo screens are provided by the present invention.

The present inventors have observed that over expression of RFC inhibitory fragments in the presence of the RelA subunit, resulted in cell death in 293 cells. This observation could be exploited to create a cell

based assay for molecules that had a similar effect. An example of a suitable screen would be as follows:

The cells used could be either for example (a) 293 cells transiently transfected with RelA or (b) 293 cells (or other cells found to support this effect) containing a chromosomally integrated RelA expression construct, such as a plasmid. This latter case would have the advantage of consistency during the assay since transient transfection conditions can vary and expression levels change depending on the time after transfection. The RelA could also be inducible, under the control of for example a tetracycline or IPTG regulated promoter. This would allow direct determination of the RelA dependency of any molecules isolated in the same cells (which would also be an advantage in terms of consistency).

The compounds to be screened could be added to cells over expressing RelA and/or control cells. The end point of the screen would be cell death (apoptotic or otherwise). The purpose would be to seek to identify molecules that killed cells in the presence of over expressed RelA but had no, or limited effect on control cells. Thus RelA dependent inducers of cell death would be isolated, mimicking the effect of RFC (p140) fragments. These would be taken for further studies and evaluation of potential clinical use.

The present inventors have observed that cell death depended on using unfiltered serum (suggesting that the NF-kB/RFC cell death effect results from increased sensitivity to a component of serum). It is preferred that that the serum used for a screen be consistent and validated for supporting this effect. Molecules that mimicked the RFC fragment cell death effect could be subsequently tested for serum dependence. Molecules that functioned in a serum independent manner may be considered as being more potent and potentially efficacious.

(2) *In vitro assay for inhibition of RelA/RFC (p140) interaction.* An explanation for the RelA dependent cell death effects of RFC (p140) fragments in vivo is that they disrupt the interaction of RelA with endogenous, full length RFC (p140). A standard immunassay such as an ELISA based assay could be provided to isolate molecules that also disrupt this interaction.

Typically a first purified protein would be immobilised on a substrate. This could be either (a) Full length RelA or the RelA amino terminal Rel Homology Domain (RHD) which has been shown to bind RFC (p140) in vitro, or (b) Full length RFC (p140) or RFC (p140) fragments 1 or 3, which are shown to interact separately with RelA. These proteins may be generated by recombinant means such as by expression in bacteria such as *Escherichia Coli* or insect such as, sf9, cells,

followed by subsequent purification by conventional means utilising for example a tag, such as GST or His.

After preferential blocking with non specific proteins (to prevent binding of proteins added subsequently to the substrate), the second partner protein would be added to the substrate. This could be in a suitable medium such as a buffered medium eg. phosphate buffered saline. This second protein would also be one of those listed above. Thus, if RelA or fragment thereof was immobilised on the plate then the second protein would be derived from RFC (p140) and vica versa.

Test molecules may be added at the same time as addition of the second protein (or alternatively be preincubated with one of the target proteins). After a period of incubation, the RelA/RFC interaction may be detected for example using an antibody to the second, non immobilised, protein. A second antibody, conjugated to a suitable detection molecule (e.g. alkaline phosphatase) would then be used to produce a signal, indicative of the presence of an interaction, which could be quantitated.

Molecules that disrupted the RelA/RFC interaction could be considered those most likely to be of clinical use. However any molecules that enhanced the interaction might also be of interest, however (protein:protein interactions in vivo are often dynamic and enhancement of an interaction might also have a clinically useful

outcome).

Further refinement of any molecules isolated in this screen could be accomplished by solving the crystallographic 3D structure of the RelA/RFC interaction (either full length proteins or fragments or with the inhibitory molecule itself) and subsequent molecular modelling.

RFC (p140) also interacts with other proteins including, but not limited to, other RFC fragments, PCNA, retinoblastoma, p53 and C/EBP alpha. Molecules isolated above could be tested in similar assays to determine specificity. In addition, screens could be devised to disrupt these other interactions. It is possible that the combination of, for example, a molecule that specifically disrupted a p53/RFC interaction and a molecule that disrupted a RelA/RFC interaction might be the most useful in a clinical setting.

Evaluation of clinical usefulness

This evaluation would initially be to determine the effect of the molecules isolated above on a wide range of cell lines. These could be tumour derived cells lines as well as those derived from inflammatory diseases. Control, "normal" cells would be used as a reference point. A positive effect would be judged as being either the induction of cell death or cell cycle arrest. It could be evaluated whether this was dependent upon NF-κB

(by using cells which have aberrently active NF-κB). Any other dependence, such as on p53 or retinoblastoma protein status, could also be determined. Later studies could involve analysis of effects in animal model systems before clinical trials were performed. It is postulated that these inhibitors could be used to treat diseases which have aberrently active NF-κB as an underlying cause and where induction of cell death or cell cycle arrest would be a desirable outcome. NF-κB can also have affects on angiogenesis or metastasis so it is possible that other effects, not testable or observable in the current laboratory assays, might also be desirable properties of these molecules. They might also be useful where current treatments activate NF-κB (e.g. chemotherapy or radiotherapy for cancer) and where this activation inhibits the effectiveness of the therapy.

Inhibitors which are identified can either be expressed in cells or applied exogenously using various techniques.

The inhibitors may be useful in the treatment of cancer, particularly breast cancer and other cancers where NF-kappaB is found to be aberrantly active. In addition to breast cancer NF-κB has been found aberrantly active in pancreatic adenocarcinoma, melanoma, head and neck squamous cell carcinoma, acute lymphoblastic leukemia, Hodgkin's lymphoma and hepatocellular

carcinoma. Moreover, NF-κB activity has been associated with the growth, angiogenesis and metastasis of human melanoma cells in nude mice. The extent of NF-κB's involvement in cancer has yet to be truly ascertained, however, and it is entirely possible that its activation will prove to be a frequent occurrence in most tumour types.

The treatment according to the present invention may advantageously be used in conjunction with traditional cancer therapies (ie. chemotherapy and radiotherapy) which have been shown to activate NF-kappaB, and where this NF-kappaB activation reduces the effectiveness of the therapy (by preventing cell death).

The inhibitors may also be useful in treatment of proliferative diseases such as occur in some skin disorders and inflammatory diseases such as rheumatoid arthritis or inflammatory bowel disease. They may also be applied to diseases caused by viruses, such as human immunodeficiency virus 1(HIV-1) which is the causative agent of AIDS, where NF-kappaB has been shown to play an important role. These compounds might also prove useful in the treatment of neurodegenerative diseases, such as Alzheimer's disease, where a role for NF-kappaB has also been indicated.

The present invention thus also extends to the use of an agent which disrupts an interaction between RelA and RFC (p140) for the manufacture of a medicament for

use in therapy such as the treatment of diseases mentioned hereinabove.

Embodiments of the invention will now be described by way of example only and with reference to the Figures which show:

Figure 1: RFC (p140) regulates RelA and p53 transactivation.

(A) U2OS cells were transfected with the 3 x κB ConA luciferase reporter plasmid (1.5 μg) and the indicated RSV RelA (1 μg) or pCDNA3 RFC (p140) (0.1, 0.5, 1 and 2 μg) expression plasmids. Control RSV or pCDNA3 plasmids were included in all transfections such that each condition had the same level of each type of plasmid. Cells were harvested after 30 hours. Results shown are the means of three separate experiments. Standard deviations are shown.

(B) U2OS cells were transfected as in (A) with A20 CAT reporter plasmid (5 μg) and the indicated RSV RelA (5 μg) or pCDNA3 RFC (p140) (0.5 μg) expression plasmids. Results shown are the means of three separate experiments. Standard deviations are shown.

(C) U2OS cells were transfected as in (A) with the Bax luciferase reporter plasmid (1.5 μg) and the indicated pCDNA3 p53 (100ng) or pCDNA3 RFC (p140) (0.5 μg) expression plasmids. Results shown are the means of

three separate experiments. Standard deviations are shown.

Figure 2: RelA and p53 interact with RFC (p140).

(A) Immunoprecipitated RelA binds *in vitro* translated RFC (p140). RelA was immunoprecipitated from nuclear protein extracts (200 µg) prepared from 293 cells transfected with a RelA expression plasmid. The immunoprecipitated complex was then used in a pull down assay with reticulocyte lysate translated RFC (p140). A sample of input material (10%) is shown in this and subsequent figures.

(B) RFC (p140) binds the Rel homology domain (RHD) of RelA. Purified GST, GST RelA (RHD) or GST RelA (428-551), expressed in *Escherichia coli* and bound to glutathione agarose, were used in a pull down assay with reticulocyte lysate translated RFC (p140).

(C) Overexpressed RelA co-immunoprecipitates with RFC (p140). Endogenous RFC (p140) was immunoprecipitated from nuclear protein extracts (200 µg) prepared from 293 cells transfected with either RSV RelA expression plasmid or a control plasmid. The immunoprecipitated complex was then resolved by SDS PAGE and immunoblotted with an anti-RelA antibody. PI = preimmune serum.

(D) Endogenous RelA co-immunoprecipitates with RFC (p140). Endogenous RFC (p140) was immunoprecipitated from U2OS cell nuclear protein extracts (300 µg) that had

been stimulated with TNF to activate endogenous NF-κB. The immunoprecipitated complex was then resolved by SDS PAGE and immunoblotted with an anti-RelA antibody.

(E) Overexpressed p53 co-immunoprecipitates with RFC (p140). Endogenous RFC (p140) was immunoprecipitated from nuclear protein extracts (200 µg) prepared from 293 cells transfected with either pCDNA3 p53 expression plasmid or a control plasmid. The immunoprecipiate complex was then resolved by SDS PAGE and immunoblotted with an anti-p53 antibody.

(F) Endogenous p53 co-immunoprecipitates with RFC (p140). Endogenous RFC (p140) was immunoprecipitated from 293 cell nuclear protein extracts (400 µg). The immunoprecipitated complex was then resolved by SDS PAGE and immuoblotted with an anti-p53 antibody.

Figure 3: RFC (p140) is specifically retained in a GST RelA (RHD) column. 293 cell nuclear protein extracts were passed over GST RelA (RHD) or GST control columns. The columns were then washed before a stepwise elution using buffer containing 75, 150, 300, 600 and 1000 mM NaCl. Eluates were TCA precipitated, resolved by SDS gel electrophoresis and analysed by western blot using the antibodies indicated. A sample of input material (10µl) is shown.

Figure 4: A dependent transactivation.

(A) Schematic diagram showing different domains of RFC (p140).

(B) Western blot analysis of RFC (p140) fragments F1-4. Nuclear extracts were prepared from 293 cells transfected with 5µg of pCGN RFC (p140) fragment expression plasmids. Samples were resolved by SDS-PAGE and immunoblotted with 12CA5 anti HA-antibody.

(C) 10cm dishes of U2OS cells were transfected with A20 CAT reporter plasmid (5 µg) and the indicated RSV RelA (5 µg) and pCGN RFC (p140) fragment (5 µg) expression plasmids. Control RSV or CMV plasmids were included in all transfections such that each condition had the same level of each type of plasmid. Cells were harvested after 30 hours and assayed for CAT activity. Results shown are the means of three separate experiments. Standard deviations are shown.

(D) 10cm dishes of U2OS cells were transfected with Gal4 E1B CAT reporter plasmid (5 µg), pCGN RFC (p140) fragments (5 µg) expression plasmids and 0.15ng of pCDNA3 Gal4, Gal4 RelA (aa 428-551) and Gal4 VP16 as indicated. Control pCDNA3 plasmids were included in all transfections such that each condition had the same level of each type of plasmid. Cells were harvested after 30 hours and assayed for CAT activity. Results shown are the means of three separate experiments. Standard deviations are shown.

Figure 5: Disruption of RFC (p140) function results in RelA dependent induction of cell death.

(A & B) 10cm dishes of 293 cells were transfected with the indicated pVR1012 RelA (5 µg in A, 0.5 µg in B) and pCGN RFC (p140) (5 µg in A, 0.5 µg in B) expression plasmids. Control pVR1012 or CMV plasmids were included in all transfections such that each condition had the same level of each type of plasmid. Representative fields of view are shown. Cell death was seen to occur between 48 and 72 hours after transfection.

(C) 293 cells were transfected as above with 5µg of expression plasmids. 72 hours after transfection cells were harvested and stained with trypan blue. Living and dead cells were then counted in triplicate using a haemocytometer. The results from two separate experiments are shown.

(D & E) 293 cells were transfected as above with 5µg of expression plasmids and whole cell lysates were prepared after 48 hours but prior to cell death occurring. Extracts were resolved by SDS-PAGE and immunoblotted with either anti-HA antibody to detect RFC protein fragment expression levels (D) or anti-RelA antibody (E).

Figure 6: siRNA mediated down regulation of RFC (p140) levels inhibits endogenous NF-κB transactivation.

(A) siRNAs directed against RFC (p140) and RelA specifically inhibit the expression of their target proteins. Western blot showing the affect on RFC (p140), RelA and β-actin proteins after treating HeLa 57A cells with the indicated siRNAs. siRNAs used were a scrambled sequence control, two anti-RFC (p140) siRNAs (A and B) and an anti RelA siRNA.

(B) The affect of siRNA treatment on stimulation of an integrated NF-κB reporter plasmid by TNF α. HeLa 57A cells containing an integrated NF-κB reporter plasmid were treated with the indicated siRNAs. Cells were either left unstimulated or were subjected to TNF α stimulation (10 ng/ml) for 6 hours as indicated. Luciferase activity is expressed as fold activation relative to the level of activity seen in unstimulated cells with the scramble siRNA control. Results are the mean of three separate experiments and standard deviations together with fold activations are shown.

(C) Treatment with anti RFC (p140) siRNA does not affect IκB α degradation and resynthesis. HeLa 57A cells were treated as in (B) and whole cell lysates were prepared and subjected to western blot analysis with the indicated antibodies.

(D) Treatment with with siRNAs does not affect cell viability. HeLa 57A cells were treated as in (B) and

were then stained with crystal violet.

Figure 7: Mapping the sites of interaction between RelA and RFC (p140)

(A) RelA interacts with RFC (p140) fragments F1 and F3. 293 cell nuclear protein extracts were prepared from cells transfected with the indicated, HA tagged, fragments of RFC (p140) or RFC (p37). Equivalent levels of protein extract, in incubation buffer (IB) (20mM Hepes, pH 7.9, 2.5mM MgCl2, 1mM DTT, 0.1% NP-40, 0.5mM PMSF, 1mg/ml leupeptin, 1mg/ml aprotinin and 1mg/ml pepstatin A) containing 75mM NaCl, were passed over 0.4ml GST RelA (RHD) or GST control columns (prewashed with 20 volumes if IB (75mM NaCl) and 2 volumes of IB (1M NaCl)). The columns were then washed with 20 volumes of IB (75mM NaCl) before a stepwise elution in 500ml of IB containing 75, 150, 300, 600 and 1000 mM NaCl. Eluates were TCA precipitated, resolved by SDS gel electrophoresis and analysed by western blot using an anti HA anitbody. A sample of input material (10ml) is shown.

(B) RFC (p140) binds the amino terminal sub domain of the RelA Rel homology domain. Purified GST RelA (1-97), GST RelA (1-196) or GST RelA (97-307), expressed in *Escherichia Coli* and bound to glutathione agarose, were

used in a pull down assay with reticulocyte lysate translated RFC (p140) or luciferase control protein as indicated.

Figure 8: RFC (p140) regulates RelA transactivation

(A,B)RFC (p140) stimulates RelA transcriptional activity. U2OS cells were transfected with the 3 x κB ConA luciferase reporter plasmid (B) or ConA luciferase control plasmis (B) (1.5 µg) and the indicated RSV RelA (1 µg) or pCDNA3 RFC (p140) (0.1, 0.5, 1 and 2 µg) expression plasmids. Control RSV or pCDNA3 plasmids were included in all transfections such that each condition had the same level of each type of plasmid. Cells were harvested after 30 hours. Results shown are the means of three separate experiments. Standard deviations are shown.

(C) RFC (p140) does not affect transfected RelA protein levels. U2OS cells were transfected as in (A) however after 30 hours, whole cell lysates were prepared and analysed by western blot analysis for RelA protein levels.

(D) RFC (p140) does not affect RelA DNA-binding. 10 cm dishes of 293 cells were transfected with RelA or RFC (p140) expression plasmids (5 µg of each) either alone or

in combination as indicated. Nuclear protein extracts were prepared and analysed by electrophoretic mobility shift assay (EMSA) using a ^{32}P labelled oligonucleotide containing the Ig/HIV NF-κB binding site. The position of the RelA/DNA complex is indicated.

Materials and Methods

Plasmids

The RSV RelA, GST RelA (RHD) and pCDNA3 Gal4 plasmids have been previously described (11, 12). The pCDNA3 p53 expression plasmid was created by subcloning the p53 cDNA from plasmid pC53-SN3. The p53 cDNA was inserted into the *Bam*HI site of the pCDNA3 polylinker. The pGL3 Bax luciferase reporter plasmid was supplied by Dr. T. Crook and originated in Dr. J. Reed's laboratory (Burnham Insititute, La Jolla, California). The pVR1012 RelA plasmid was obtained from Professor Gary Nabel (NIH). pVR1012 is used with the permission of Vical Inc. The full length RFC (p140) cDNA was isolated from human foreskin fibroblast cell RNA by RT PCR and inserted into the *Kpn*I site of pCDNA3 or pCGN, which inserted an HA tag at its amino terminus. Fragments of RFC (p140) were isolated by PCR from the original clone and also inserted into the *Kpn*I site of pCGN. The A20 CAT reporter plasmid has been described previously (13). The 3 x κB ConA

luciferase reporter plasmid was provided by Professor Ron Hay (University of St. Andrews).

Antibodies

The polyclonal RFC (p140) antibody was generated using a purified, His tagged, fragment of the protein (aa 1-369) expressed in *Escherichia coli*. The antibody was raised in sheep by the Scottish Antibody Production Unit (SAPU). RelA(p65) western blots and immunoprecipitations were performed with Santa Cruz Biotechnology antibodies sc-372 and sc-109 respectively. p300 western blots were performed with Pharmingen antibody 14991A. The Rad 50 antibody was obtained from GeneTex (MS-RAD10-PX1). The Rb, MSH2 and 6 antibodies were obtained from Santa Cruz Biotechnology (sc-50, sc-494 and sc-1243 respectively). The PC10 anti PCNA monoclonal antibody was purchased from Sigma. The HA tag antibody was obtained from Dr. Barbara Spruce (Dundee).

Transfections and reporter assays

Calcium phosphate transfections of U2OS and 293 cells have been described previously (14). In this study, U2OS cells were split the night before transfections took place and harvested after 30 hours. 293 cells were split 2 hours before transfection and harvested/analysed after 48 hours. 293 cells for cell death studies were grown in DMEM media with 10% unfiltered fetal bovine serum. Transfections for CAT and

luciferase assays were performed using 10 and 6 cm dishes respectively. CAT activity was assayed on 10-100µg of protein prepared from whole cell lysates. For luciferase assays, lysates were prepared using passive lysis buffer (Promega). Luciferase assays were performed according to manufacturer's instructions (Promega). All experiments were performed separately, a minimum of three times before calculating means and standard errors as shown in figures. Relative luciferase levels were calculated as the level of activity seen per µg of protein extract. Internal control reference plasmids (such as those encoding β galactosidase or renilla luciferase) were not included. When investigating transcription factor function, the promoters driving the expression of such internal controls are often affected by other components of the experiment and can lead to the incorporation of errors when data is calculated relative to their levels of expression.

Nuclear protein extracts

Nuclear protein extracts were prepared essentially by the method of Dignam, except in Fig 2D where nuclei were extracted in 150mM NaCl.

GST pull downs and immunoprecipitations

In vitro pull down assays and immunoprecipitations were performed as described previously (11, 15).

Affinity chromatography

293 cell nuclear protein extracts, in incubation buffer (IB) (20mM Hepes, pH 7.9, 2.5mM $MgCl_2$, 1mM DTT, 0.1% NP-40, 0.5mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin and 1µg/ml pepstatin A) containing 75mM NaCl, were passed over 0.4ml GST RelA (RHD) or GST control columns (prewashed with 20 volumes if IB (75mM NaCl) and 2 volumes of IB (1M NaCl)). The columns were then washed with 20 volumes of IB (75mM NaCl) before a stepwise elution in 500µl of IB containing 75, 150, 300, 600 and 1000 mM NaCl. Eluates were TCA precipitated, resolved by SDS gel electrophoresis and analysed by western blot.

Procedures

To establish whether RFC (p140) might regulate NF-κB the present inventors initially investigated whether the former might have any effect on RelA transcriptional activity. U2OS cells were transfected with RFC (p140) and RelA expression plasmids together with a 3x κB luciferase reporter plasmid, which contains three copies of the consensus Ig/HIV NF-κB binding site upstream of a minimal ConA promoter. A significant and dose dependent increase of RelA transactivation was observed (Fig. 1A), comparable to that typically seen with the p300 protein, a known coactivator of NF-κB (15). The inventors next investigated whether a similar effect would be seen with a cellular promoter known to regulated by NF-κB. The A20

protein is an inhibitor of apoptosis whose promoter contains two NF-κB binding sites (13). Upon co-transfection with the A20 CAT reporter plasmid, full length RFC (p140) again significantly enhanced RelA transactivation, although to a lesser extent than that seen with the artificial promoter (Fig. 1B). These observations were consistent with previous reports suggesting that RFC (p140) could function as a coactivator protein (16). We were interested therefore in whether RFC (p140) would stimulate the transactivation function of any transcription factor. To investigate this, the inventors examined the effect of RFC (p140) on the p53 tumour suppressor protein. In contrast to the results seen with RelA, however, RFC (p140) repressed p53 transactivation of the Bax promoter (Fig. 1C). RFC (p140) is not a universal coactivator protein, therefore. Moreover, stimulation of RelA and repression of p53 is consistent with both the pro-proliferative and anti-apoptotic functions of RFC (p140).

Stimulation of RelA transactivation by RFC (p140) could conceivably occur through a number of different mechanisms. Some of these, such as indirect cell cycle effects or interactions with other coactivators, would not require a direct interaction between the two proteins. The inventors next determined, therefore, whether RelA and RFC (p140) could physically associate with each other. Significantly, both immunoprecipitated

RelA complexes and bacterially expressed GST RelA, bound to reticulocyte lysate translated RFC (p140) (Fig. 2A & B). This interaction was mediated by the amino terminal RHD of RelA, which has been shown to bind many heterologous transcription factors and coactivators (11, 17, 18). This interaction was not significantly affected by the inclusion of ethidium bromide (to disrupt protein DNA interactions), indicating that it does not result from fortuitous co-localisation on the same DNA fragment during incubation (data not shown). Confirming the significance of this *in vitro* interaction, transiently transfected RelA was seen to co-immunoprecipitate with endogenous RFC (p140) (Fig. 2C). p300, a known RelA coactivator (15), did not co-precipitate with the RFC (p140)/RelA complex, suggesting these proteins independently regulate NF-κB activity (Fig. 2C). Importantly, endogenous RelA and RFC (p140) in nuclear extracts prepared from TNF stimulated U2OS cells were also seen to associate (Fig. 1D). Interestingly, both overexpressed and endogenous p53 also co-immunoprecipitated with RFC (p140) (Fig. 2E & F). A larger quantity of nuclear protein extract was used in Fig. 2F relative to Fig. 2E and the membrane was exposed to film for a longer period in order to detect the interaction between the endogenous proteins. While these results, together with those from Fig. 1, suggest a real and important interaction between p53 and RFC (p140), the

rest of this report focuses on the significance and consequences of the interaction with RelA.

RFC (p140) can associate with a number of other cellular proteins. To determine if these could also bind RelA, nuclear protein extracts, prepared from unstimulated 293 cells, were passed over a GST RelA (RHD) affinity column. As expected, RFC (p140) was retained on the column (Fig. 3). No interaction was seen with Rb, PCNA and components of the BASC complex such as MSH2, MSH6 and Rad50 (Fig. 3). Although it cannot be excluded that these proteins might associate with RelA through RFC (p140), this experiment demonstrates both the specificity of this interaction and the potential for RFC (p140) to independently regulate NF-κB.

To study the role of RFC (p140) in RelA induced gene expression further, the inventors investigated whether disrupting endogenous RFC (p140) function would affect RelA transcriptional activity. To perform these experiments, a series of plasmids encoding RFC (p140) fragments fused to the HA epitope, which might be expected to function as dominant negative inhibitors, were constructed (Fig. 4A). RFC (p140) F1 (amino acids 1-369) encodes the amino terminus of the protein and has been shown to have a PCNA binding domain (19). RFC (p140) F2 (amino acids 367-493) encodes a domain with homology to DNA ligases and has a BRCT domain, also found in BRCA1 and other proteins involved in DNA-repair (20).

RFC (p140) F3 (amino acids 480-882) also binds PCNA, contains the domain homologous to other RFC subunits, has an LXCXE motif required for binding to Rb together with a caspase 3 cleavage site (20, 21, 22). RFC (p140) F3 has been previously shown to function as a dominant negative inhibitor of DNA replication in U2OS cells (23). RFC (p140) F4 (amino acids 728-1148) contains a domain required for association with other RFC subunits and also has two caspase 3 cleavage sites (20, 22). Western blot analysis, using an anti HA antibody, demonstrated that all RFC fragments were expressed equivalently, apart from RFC (p140) F4 where levels were slightly reduced (Fig. 4B).

In contrast to the results seen with full length RFC (p140) (Fig. 1), and consistent with a dominant negative role, RFC (p140) F1 and F3 were both strong repressors of RelA transactivation (Fig. 4C). In contrast RFC (p140) F2 and F4 had no effect on RelA transactivation. Demonstrating the specificity of these effects, no inhibition by these RFC (p140) fragments was seen with Gal4 VP16 or Gal4 RelA (aa 428-551) (Fig. 4D). Gal4 RelA (aa 428-551) contains the RelA carboxy terminal transactivation domain but lacks the Rel homology domain seen to interact with RFC (p140) in Fig. 2B.

The inventors next investigated whether coexpression of RFC (p140) or the RFC (p140) fragments, together with RelA might have a cooperative effect on proliferation and cell viability. To perform these experiments, both

proteins were over expressed in highly transfectable 293 cells. Initially, no effect was seen, however. Routinely, the fetal bovine serum used to grow these cells (and those used in the experiments described above) was filtered through a 0.2 μm membrane prior to use. It had observed elsewhere, however, that some cell types were more susceptible to stimuli inducing cell death when cultured in unfiltered serum (data not shown). These experiments were repeated, therefore, with 293 cells cultured in unfiltered fetal bovine serum. There was still no observable effect on cell viability when full length RFC (p140) and RelA, either alone or in combination, were expressed in 293 cells (Fig. 5A & B). Similarly, when expressed alone, none of the RFC (p140) fragments significantly affected cell viability (Fig. 5A-C). When co-expressed with RelA, however, a dramatic increase in cell death was observed with RFC (p140) F1 and F3 (Fig. 5A-C). Morphologically, these dead cells had the appearance of having undergone apoptosis, being shrunken and fragmented. These effects were seen over a range of transfected plasmid concentrations (ten fold less plasmid is used in Fig. 5B versus Fig. 5A). Moreover, no significant effect on RelA or RFC (p140) F1-F4 protein level is seen when these proteins are co-transfected (Fig. 5D & E). This effect on cell viability therefore represents a true co-operative effect and does not result from toxic effects resulting from over-

expression of either protein alone.

Discussion

In these examples, the present inventors have demonstrated that RFC (p140) interacts with and regulates the transcriptional activity of both RelA and p53. While RFC (p140) stimulates the activity of RelA it represses the activity of p53, however (Fig. 1). Under many circumstances both proteins have contrasting functions with RelA being pro-proliferative and anti-apoptotic while p53 is anti-proliferative and pro-apoptotic (24, 25). This observation would therefore be consistent with the reported anti-apoptotic and pro-proliferative function of RFC (p140). By interacting with RelA and p53, RFC (p140) can directly link the processes of DNA-replication and repair with apoptosis. NF-κB regulates a large number of genes in many cell types with great selectivity. The diversity of these genetic programs, and their often apparently contradictory cellular effects, has suggested that interactions with other proteins might provide part of the mechanism through which this specificity can be achieved. NF-κB has been previously linked with cell cycle regulation and can directly stimulate proliferation via the activation of proto-oncogenes such as c-Myc and cyclin D1 (26, 24). Conversely, NF-κB function is also sometimes associated with cellular differentiation and the cyclin dependent

kinase inhibitor $p21^{WAF1/CIP1}$ can stimulate RelA transactivation indirectly through the p300 and CBP coactivator proteins (15, 27). This study therefore provides an additional and direct link between NF-κB and the proteins that control cell division that, in addition to the effects on cell death seen here, might also influence RelA's ability to selectively regulate the cell cycle.

While we cannot rule out that indirect effects of RFC (p140) contribute to the regulation of RelA and p53 transactivation, the physical association between these proteins suggests that they are mediated at least in part by direct effects on transcriptional activity. How RFC (p140) accomplishes this, is currently not known, however. A number of proteins, including p53, BRCA1, TFIIH and p300, have multiple roles in transcription, replication and DNA repair (28, 29, 30, 31). Recently, RFC (p140) has also been found to interact with and stimulate transactivation by C/EBPα (16). By interacting with Rb (21), RFC (p140) could also be expected to influence the function of transcription factors such as E2F. It is possible that RFC (p140), similar to p300 and CBP, might regulate the function of many DNA-binding proteins. Interestingly, while both RelA and p53 interact with p300, no p300 was observed co-precipitating with the RelA/RFC (p140) complex, suggesting a distinct regulatory function (Fig. 2).

It was also found that disruption of RFC (p140) function can result in RelA-dependent cell death (Fig. 5). A role for RFC (p140) as a regulator of programmed cell death has been previously suggested (20, 21, 22). Moreover, RFC (p140) is a substrate for caspases and is proteolytically cleaved during apoptosis (22). The present data is consistent with full length RFC (p140) having an anti-apoptotic function, which is disrupted by the expression of dominant negative inhibitory fragments. It will be interesting to determine whether phosphorylation or other modifications of RFC (p140), leading to disruption of its normal function, might be a trigger for apoptosis to occur under some conditions.

Surprisingly, RelA induced cell death correlates with the ability of dominant negative RFC (p140) fragments to inhibit its transcriptional activity (Fig. 4). Furthermore, it was also found that to observe this effect on cell viability, it was important not to filter the fetal bovine serum used to grow the cells. These effects on cell death were observed with multiple batches of serum purchased from different companies (data not shown), indicating that this is not an aberrant result.

This observation suggests that co-expression of RelA and RFC (p140) F1 or F3 sensitises the cells to a factor present in the unfiltered serum. This sensitisation cannot be due to just the repression of NF-κB activity by the RFC (p140) fragments since a strong co-operative

effect is seen. It is possible that under these conditions, RelA is still capable of inducing (or repressing) the expression of selective endogenous genes, some of which will be capable of facilitating an apoptotic response. Alternatively, expression of RelA and the RFC (p140) fragments might effect the activity of other transcription factors, which can then also contribute to this effect. Nonetheless, and although these observations are based on transcription factor overexpression, this experiment does reveal a novel pathway through which RelA and RFC (p140) regulate cell viability.

Aberrant activation of RelA is increasingly associated with many forms of cancer where its anti-apoptotic activity contributes towards the process of tumorigenesis (32). Furthermore, inhibition of apoptosis by RelA can reduce the effects of many chemotherapeutic drugs (33). The present results imply that disruption of RFC (p140) might also represent a valid strategy for the treatment of cancer. Targeting RFC (p140) would not only inhibit cellular proliferation but by switching RelA function to being pro-apoptotic, tumour cells might specifically be induced to undergo apoptosis.

Further experimental details

(1) siRNA experiments

Double stranded RNA molecules homologous to specific genes (siRNAs also known as RNAi or interfering RNA) have recently been shown to result in the down regulation of their target full length mRNAs in human cells.

siRNA (RNAi) oligonucleotides (double stranded RNA oligonucleotides) have been used to investigate the function of endogenous RFC (p140) in HeLa 57A cells (these HeLa cells contain a chromosomally integrated copy of the 3 x κB ConA luciferase reporter plasmid used in transient transfection studies (see section 4 below). These cells were obtained from Prof. Ron Hay, University of St Andrews (no MTA required). Transfection of siRNAs, homologous to a specific gene, into cell lines results in the specific down regulation of target mRNA and protein levels.

Using two different siRNAs directed against RFC (p140) with a scramble siRNA and RelA siRNA as controls, we have shown specific down regulation of endogenous RFC (p140) protein in HeLa 57A cells (Fig. 6A).

RFC (p140) siRNA sequences (sense strand only)

RFC (p140) A: GAAGGCGGCCUCUAAAUCA

RFC (p140) B: UGAUGAAGCCAUCGCCAAG

Control siRNA sequences used in the study

RelA: GCUGAUGUGCACCGACAAG

"Scramble": CAGUCGCGUUUGCGACUGG

The primers were designed according to the guidelines at the following web site

http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html

This provides more detail for the information provided in this reference

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494-498.

The siRNA sequences can essentially be homologous to any part of the mRNA. Since the whole protein becomes down regulated there is no need to target a domain. The rule for selecting them is to find an AA sequence (although it appears this might not be absolutely required) followed by 19 nulceotides with approximately 50% GC content. So in a large protein there are many possible sequences.

Using the HeLa 57A cells we have shown that down regulation of RFC (p140) results in a significant inhibition of Tumour Necrosis Factor (TNF) alpha mediated activation of this reporter. TNF is a well established activator of endogenous NF-κB and our siRNA directed to the RelA(p65) NF-κB subunit also causes a more potent

(but still comparable) inhibition of this activation (Fig. 6B).

IκB α degradation and resynthesis following TNF stimulation are unaffected in RFC (p40) siRNA treated cells (Fig. 6C). This suggests that NF-κB activation is not affected by the RFC (p140) siRNA. It also indicates that not all NF-κB target genes are affected by loss of RFC (p140) synthesis since IκB α resynthesis is NF-κB dependent.

These effects do not result from cell death. Cells treated in exactly the same way as those harvested for reporter gene assays show no defects in proliferation or cell death as judged by crystal violet staining (Fig. 6D). As seen in our previous 293 cell cotransfection experiments (in original patent application), inhibition of RFC (p140) by co-expression of dominant negative fragments does not appear to be intrinsically toxic to cells. This suggests that the role RFC (p140) has been previously shown to perform as a replication factor can be compensated for by other proteins or can still occur with residual levels of the protein left after siRNA treatment (inhibition is not 100%). This suggests that a putative pharmacological inhibitor of RFC (p140) or an NF-κB/RFC (p140) interaction might not be intrinsically toxic to cells and would function specifically. The time of TNF treatment in these experiments is not sufficient to see induced cell death effects emerge, however.

These experiments are very significant. Previous functional results had relied on over expression of RFC (p140), RFC (p140) fragments, the RelA NF-κB subunit and (apart from cell death experiments) transiently transfected NF-κB reporter plasmids. Here it has been demonstrated that endogenous RFC (p140) regulates endogenous NF-κB with an integrated reporter. siRNAs directed against RFC (p140) could therefore potentially be used as agents to manipulate RFC and NF-κB function in vivo. This data also suggests that other small molecular inhibitors that disrupt RFC (p140) function directly (i.e. are not specifically designed just to disrupt the interaction between RFC (p140) and RelA) could also be useful tools to manipulate the NF-κB response.

(2) Mapping the site of interaction

It has been demonstrated that a protein affinity column consisting of a glutathione s-transferase (GST) fusion to the RelA amino terminus (the first 304 amino acids comprising the rel homology domain (RHD)) could interact with full length RFC (p140) when a HeLa cell nuclear protein extract was passed over it. Using this technique, we have now shown that the fragments of RFC (p140) F1 and F3, interact independently with the RelA RHD while F2 and another subunit of RFC (RFC (p37)) do not (Fig. 7A). In this experiment, the cDNAs encoding

these protein fragments were epitope tagged and over expressed in 293 cells. Nuclear extracts were made from these transfected cells and then passed over the GST RelA affinity column. Bound proteins were eluted with a stepwise salt gradient and analysed by western blotting.

(1) Fragments of RFC (p140).

The inventors discovered that two fragments of RFC (p140), designated F1 and F3, affect RelA(p65) function as follows.

(a) RFC F1 and F3 inhibit RelA transcriptional activation in a transient transfection assay with an NF-κB reporter plasmid in U2-OS cells.

(b) RFC F1 and F3 synergise with RelA to induce cell death in 293 cells.

Both of these RFC fragments can separately interact with RelA (see Fig. 7). Without wishing to be bound by theory it is probable, therefore, that they function by inhibiting the interaction of RelA with endogenous, full length RFC (p140). It is also probable that they can also function as dominant negative inhibitors of other RFC (p140) functions and F3 has been previously shown to be such an inhibitor (34).

These RFC fragments (or derivatives such as smaller versions that do the same thing) could therefore be potentially used as therapeutic agents to manipulate the NF-κB response in vivo (possibly when expressed in

adenoviral vectors).

This experiment is significant since we had previously shown that RFC (p140) fragments F1 and F3 were the fragments that inhibited NF-κB transcriptional activity and also synergised with RelA to induce cell death. These new results indicate that these fragments both independently interact with RelA. This suggests that the functional effects that we observe with these fragments derive, at least in part, from an ability of both fragments to disrupt the interaction between RelA and endogenous RFC (p140).

The inventors have also found that the first, most amino terminal Sub domain (amino acids 1-196) of the RelA Rel Homology Domain (RHD) is sufficient for the interaction with full length RFC (p140) in vitro (Fig. 7B).

In addition the inventors have performed a number of experiments as controls for the original data presented.

(a) It has have established that full length RFC (p140) stimulates RelA transcriptional activity using a generic NF-κB reporter containing multiple NF-κB binding sites (3 x κB luciferase) in transient transfection experiments in U-2 OS cells (Fig. 8A). The original data just used the promoter from the NF-κB regulated A20 gene. The effects seen with this new reporter are more pronounced than with the A20 promoter. It has also been

found that a control reporter plasmid (same plasmid backbone as the 3 x κB luciferase plasmid but lacking the NF-κB binding sites) is not activated by RFC (p140) (Fig. 8B). These results show that the effects of RFC (p140) are specific. It has been shown that co-transfection of RFC (p140) does not affect RelA expression levels (Fig. 8C) or DNA-binding in an electrophoretic mobility shift assay (EMSA) (Fig. 8D). These data support the idea that RFC (p140) stimulates RelA transcriptional activity.

REFERENCES

1. Perkins, N.D. The Rel/NF-kappaB family: friend and foe. *Trends Biochem. Sci.* 25, 434-440 (2000).

2. Pahl, H.L. Activators and target genes of Rel/NF-kappa B transcription factors. *Oncogene* 18, 6853-6866 (1999).

3. Perkins, N.D. Achieving transcriptional specificity with NF-kappa B. *International Journal Of Biochemistry & Cell Biology* 29, 1433-1448 (1997).

4. Barkett, M. & Gilmore, T.D. Control of apoptosis by Rel/NF-kappa B transcription factors. *Oncogene* 18, 6910-6924 (1999).

5. Mossi, R. & Hubscher, U. Clamping down on clamps and clamp loaders - The eukaryotic replication factor C. *European Journal of Biochemistry* 254, 209-216 (1998).

6. Wang, Y. *et al.* BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes & Development* 14, 927-939 (2000).

7. Pennaneach, V. *et al.* The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent manner. *Molecular Cell* 7, 715-727 (2001).

8. Harbour, J.W. & Dean, D.C. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & Development* 14, 2393-2409 (2000).

9. Zhang, H.B. *et al.* BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* 16, 1713-1721 (1998).

10. Scully, R. *et al.* BRCA1 is a component of the RNA polymerase II holoenzyme. *Proceedings of the National Academy of Sciences of the United States of America* 94, 5605-5610 (1997).

11. Chapman, N.R., and N.D. Perkins. 2000. Inhibition of the RelA(p65) NF-kB subunit by Egr-1. J. Biol. Chem. 275:4719-4725.

12. Perkins, N.D., A.B. Agranoff, E. Pascal, and G.J. Nabel. 1994. An interaction between the DNA-binding domains of RelA(p65) and Sp1 mediates human immunodeficiency virus gene activation. Mol.Cell.Biol. 14:6570-6583.

13. Laherty, C.D., N.D. Perkins, and V.M. Dixit. 1993. Human T Cell Leukemia Virus Type I Tax and Phorbol 12-Myristate 13-Acetate Induce Expression of the A20 Zinc Finger Protein by Distinct Mechanisms Involving Nuclear Factor kB. J. Biol. Chem. 268:5032-5039.

14. Webster, G.A., and N.D. Perkins. 1999. Transcriptional cross talk between NF-kB and p53. Mol. Cell. Biol. 19:3485-3495.

15. Perkins, N.D., L.K. Felzien, J.C. Betts, K.Y. Leung, D.H. Beach, and G.J. Nabel. 1997. Regulation of NF-kB by cyclin-dependent kinases associated with the p300 coactivator. Science 275:523-527.

16. Hong, S.H., S.J. Park, H.J. Kong, J.D. Shuman, and J.H. Cheong. 2001. Functional interaction of bZIP proteins and the large subunit of replication factor C in liver and adipose cells. J. Biol. Chem. 276:28098-28105.

17. Perkins, N.D. 1997. Achieving transcriptional specificity with NF-kB. Int. J. Biochem. Cell B. 29:1433-1448.

18. Zhong, H.H., R.E. Voll, and S. Ghosh. 1998. Phosphorylation of NF-kB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol. Cell 1:661-671.

19. Montecucco, A., R. Rossi, D.S. Levin, R. Gary, M.S. Park, T.A. Motycka, G. Ciarrocchi, A. Villa, G. Biamonti, and A.E. Tomkinson. 1998. DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. EMBO J. 17:3786-3795.

20. Mossi, R., and U. Hubscher. 1998. Clamping down on clamps and clamp loaders - The eukaryotic replication factor C. Eur. J. Biochem. 254:209-216.

21. Pennaneach, V., I. Salles-Passador, A. Munshi, H. Brickner, K. Regazzoni, F. Dick, N. Dyson, T.T. Chen, J. Y.J. Wang, R. Fotedar, and A. Fotedar. 2001. The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent

manner. Mol. Cell 7:715-727.

22. Rheaume, E., L.Y. Cohen, F. Uhlmann, C. Lazure, A. Alam, J. Hurwitz, P.P. Sekaly, and F. Denis. 1997. The large subunit of replication factor C is a substrate for caspase- 3 in vitro and is cleaved by a caspase-3-like protease during Fas- mediated apoptosis. EMBO. J. 16:6346-6354.

23. Fotedar, R., R. Mossi, P. Fitzgerald, T. Rousselle, G. Maga, H. Brickner, H. Messier, S. Kasibhatla, U. Hubscher, and A. Fotedar. 1996. A Conserved Domain Of the Large Subunit Of Replication Factor-C Binds PCNA and Acts Like a Dominant-Negative Inhibitor Of DNA-Replication In Mammalian-Cells. EMBO. J. 15:4423-4433.

24. Perkins, N.D. 2000. The Rel/NF-kB family: friend and foe. Trends Biochem. Sci. 25:434-440.

25. Vousden, K.H. 2000. p53: Death Star. Cell 103:691-694.

26. Pahl, H.L. 1999. Activators and target genes of Rel/NF-kB transcription factors. Oncogene 18:6853-6866.

27. Snowden, A.W., L.A. Anderson, G.A. Webster, and N.D. Perkins. 2000. A novel transcriptional repression domain mediates p21(WAF1/CIP1) induction of p300 transactivation. Mol. Cell. Biol. 20:2676-2686.

28. Deng, C.X., and S.G. Brodie. 2000. Roles of BRCA1 and its interacting proteins. Bioessays 22:728-737.

29. Frit, P., E. Bergmann, and J.M. Egly. 1999. Transcription factor IIH: A key player in the cellular

response to DNA damage. Biochimie 81:27-38.

30. Hasan, S., P.O. Hassa, R. Imhof, and M.O. Hottiger. 2001. Transcription coactivator p300 binds PCNA and may have a role in DNA repair synthesis. Nature 410:387-391.

31. May, P., and E. May. 1999. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene 18:7621-7636.

32. Rayet, B., and C. Gelinas. 1999. Aberrant rel/nfkb genes and activity in human cancer. Oncogene 18:6938-6947.

33. Baldwin, A.S. 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J. Clin. Invest. 107: 241-246.

34. Fotedar et al. 1996. A Conserved Domain Of the Large Subunit of Replication Factor-C Binds PCNA and Acts Like a Dominant-Negative Inhibitor Of DNA-Replication In Mammalian-Cells. *EMBO. J.* 15: 4423-4433.

CLAIMS

1) An inhibitor of RFC (p140) activity for the treatment of a medical condition by the inducement of apoptosis of cells involved in the medical condition.

2) The inhibitor according to claim 1 wherein the inhibitor inhibits the interaction between RFC (p140) and RelA.

3) The inhibitor according to claim 1 or 2 wherein the inhibitor inhibits the activity of a complex between RFC (p140) and RelA.

4) The inhibitor according to claim 1 wherein the inhibitor is an inhibitory fragment of RFC (p140).

5) The inhibitor according to claim 4 wherein the inhibitor is an inhibitory fragment of RFC (F1) or RFC (F3), or derivatives thereof.

6) The inhibitor according to any one of claims 1 to 3 wherein the inhibitor is an SiRNA nucleic acid corresponding to a portion of the RFC (p140) mRNA sequence or RelA mRNA sequence.

7) The inhibitor according to claim 6 wherein the SiRNA nucleic acid consists essential of the sequence (sense strand only):

GAAGGCGGCCUCUAAAUCA or

UGAUGAAGCCAUCGCCAAG

8) The inhibitor according to any preceding claim wherein the medical condition is cancer, particularly breast cancer.

9) The inhibitor according to any one of claims 1 - 7 wherein the medical condition is a proliferative disease, particularly a proliferative skin disease.

10) The inhibitor according to any one of claims 1 - 7 wherein the medical condition is an inflammatory disease, particularly rheumatoid arthritis or inflammatory bowel disease.

11) The inhibitor according to any one of claims 1 - 7 wherein the medical condition results from viral infection, such as by human immunodeficiency virus.

12) The inhibitor according to any one of claims 1 - 7 wherein the medical condition is a neurodegenerative disease, such as Alzheimer's disease.

13) A pharmaceutical composition which comprises an inhibitor according to any one of claims 1 - 7 and a pharmaceutically acceptable carrier.

14) Use of any inhibitor according to any one of claims 1 - 7 for the manufacture of a medicament for use in therapy.

15) A method of screening for an agent for inducing apoptosis, which comprises assessing a compound for its ability to inhibit RFC (p140).

16) A method of identifying an inhibitor capable of inhibiting an interaction between RFC (14) and RelA, or an activity of a complex between RFC (p140) and RelA, comprising the steps of:

a) providing a mammalian cell capable of expressing RelA;

b) expressing RelA; and

c) adding a test compound to said cell and observing whether or not said test compound causes cell death to occur.

17) The method according to claim 16 further comprising the step of adding said compound to a control cell not over-expressing RelA and comprising an effect of said

compound on cell death of said cell with the effect on the cell expressing RelA.

18) The method according to claims 17 or 18 wherein said cell expressing RelA is a 293 cell transiently transfected with RelA or a 293 cell containing a chromosomally integrated RelA expression construct.

19) The method according to claim 18 wherein expression of RelA is under the control of an inducible promoter.

20) The method according to any of claims 16 to 19 wherein the cells is present in unfiltered serum.

21) An in vitro assay for detecting inhibition of RelA/RFC (p140) interaction comprising the steps of:

a) contacting RelA or RFC (p140), an F1 or F3 fragment thereof with a substrate;

b) optionally blocking with non-specific proteins;

c) adding RFC (p140), F1 or F3, or RelA respectively under condition which would allow interaction with the corresponding protein; and

d) adding a test compound at the same time as step c), or subsequently and observing any effect said compound has on an interaction between RelA and RFC (p140), or said F1 or F3 fragment thereof.

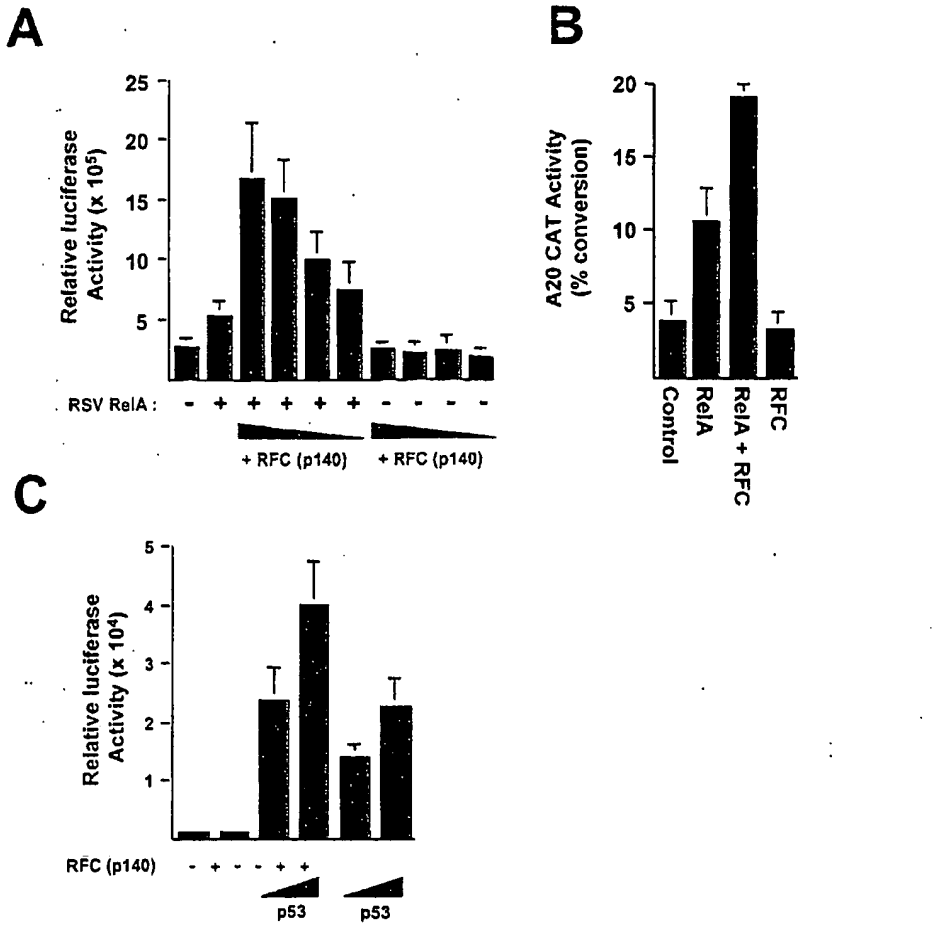

A
Relative luciferase Activity (x 10⁵)
25
20
15
10
5
RSV RelA : - + + + + + - - - -
+ RFC (p140)
+ RFC (p140)
B
A20 CAT Activity (% conversion)
20
15
10
5
Control
RelA
RelA + RFC
RFC
C
Relative luciferase Activity (x 10⁴)
5
4
3
2
1
RFC (p140) - + - - + +
p53
p53

Figure 1

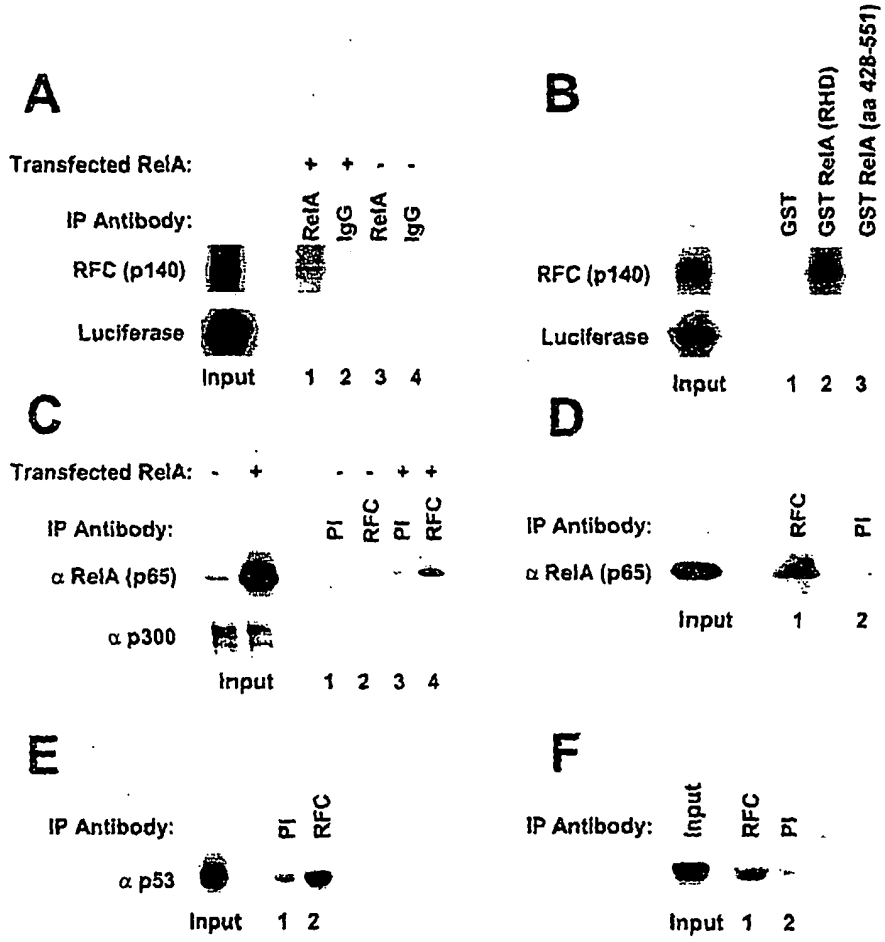

A
Transfected RelA: + + - -
IP Antibody: RelA IgG RelA IgG
RFC (p140)
Luciferase
Input 1 2 3 4
B
GST
GST RelA (RHD)
GST RelA (aa 428-551)
RFC (p140)
Luciferase
Input 1 2 3
C
Transfected RelA: - + - - + +
IP Antibody: PI RFC PI RFC
α RelA (p65)
α p300
Input 1 2 3 4
D
IP Antibody: RFC PI
α RelA (p65)
Input 1 2
E
IP Antibody: PI RFC
α p53
Input 1 2
F
IP Antibody: Input RFC PI
Input 1 2

Figure 2

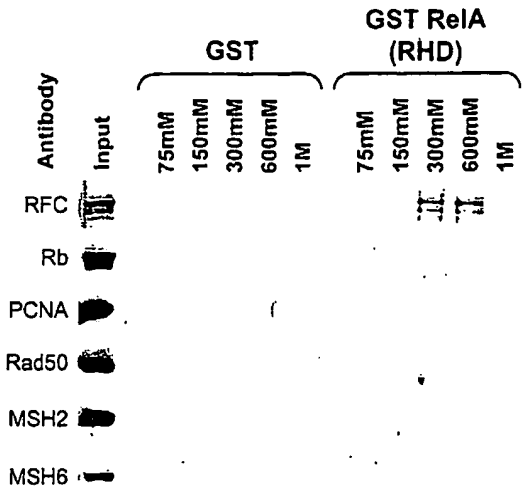
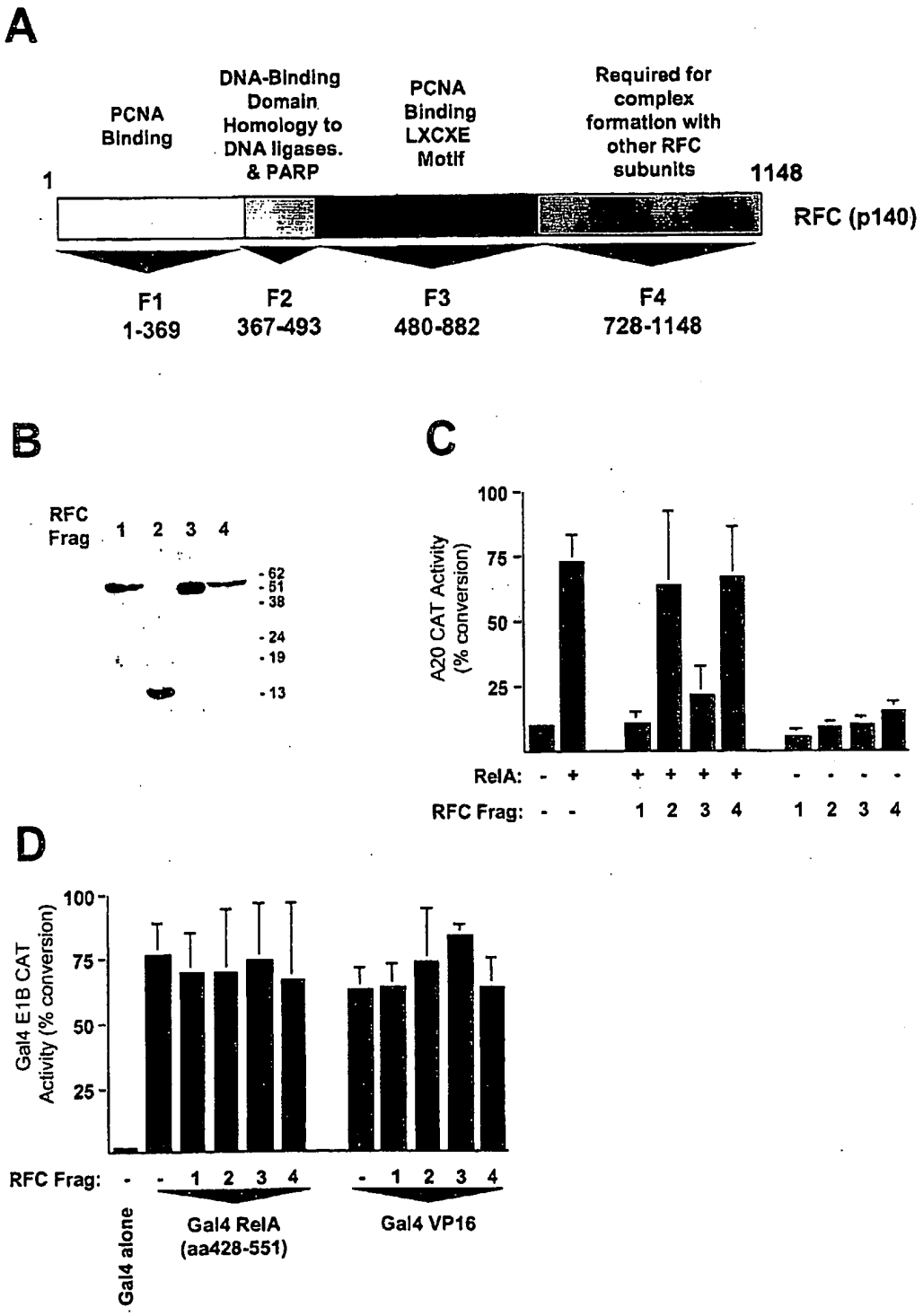

GST
GST RelA (RHD)
Antibody
Input
75mM
150mM
300mM
600mM
1M
75mM
150mM
300mM
600mM
1M
RFC
Rb
PCNA
Rad50
MSH2
MSH6

Figure 3

Figure 4


A
PCNA Binding
DNA-Binding Domain Homology to DNA ligases. & PARP
PCNA Binding LXCXE Motif
Required for complex formation with other RFC subunits
1
1148
RFC (p140)
F1 1-369
F2 367-493
F3 480-882
F4 728-1148
B
RFC Frag 1 2 3 4
62
51
38
24
19
13
C
A20 CAT Activity (% conversion)
100
75
50
25
RelA: - + + + + + - - - -
RFC Frag: - - 1 2 3 4 1 2 3 4
D
Gal4 E1B CAT Activity (% conversion)
100
75
50
25
RFC Frag: - - 1 2 3 4 - 1 2 3 4
Gal4 alone
Gal4 RelA (aa428-551)
Gal4 VP16

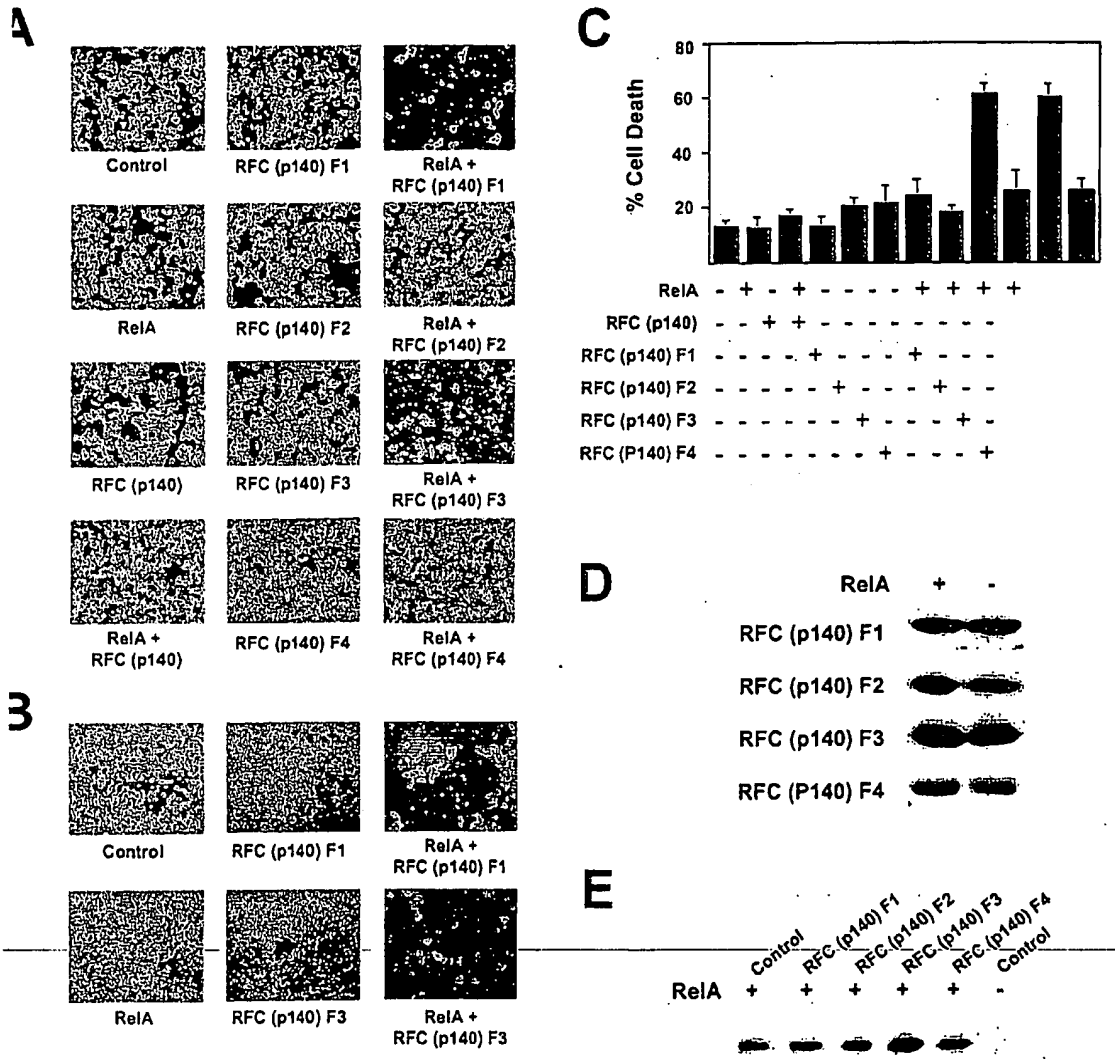

A
Control
RFC (p140) F1
RelA + RFC (p140) F1
RelA
RFC (p140) F2
RelA + RFC (p140) F2
RFC (p140)
RFC (p140) F3
RelA + RFC (p140) F3
RelA + RFC (p140)
RFC (p140) F4
RelA + RFC (p140) F4
B
Control
RFC (p140) F1
RelA + RFC (p140) F1
RelA
RFC (p140) F3
RelA + RFC (p140) F3
C
% Cell Death
80
60
40
20
RelA - + - + - - - - + + + +
RFC (p140) - - + + - - - - - - - -
RFC (p140) F1 - - - - + - - - + - - -
RFC (p140) F2 - - - - - + - - - + - -
RFC (p140) F3 - - - - - - + - - - + -
RFC (P140) F4 - - - - - - - + - - - +
D
RelA + -
RFC (p140) F1
RFC (p140) F2
RFC (p140) F3
RFC (P140) F4
E
Control
RFC (p140) F1
RFC (p140) F2
RFC (p140) F3
RFC (p140) F4
Control
RelA + + + + + -

Figure 5

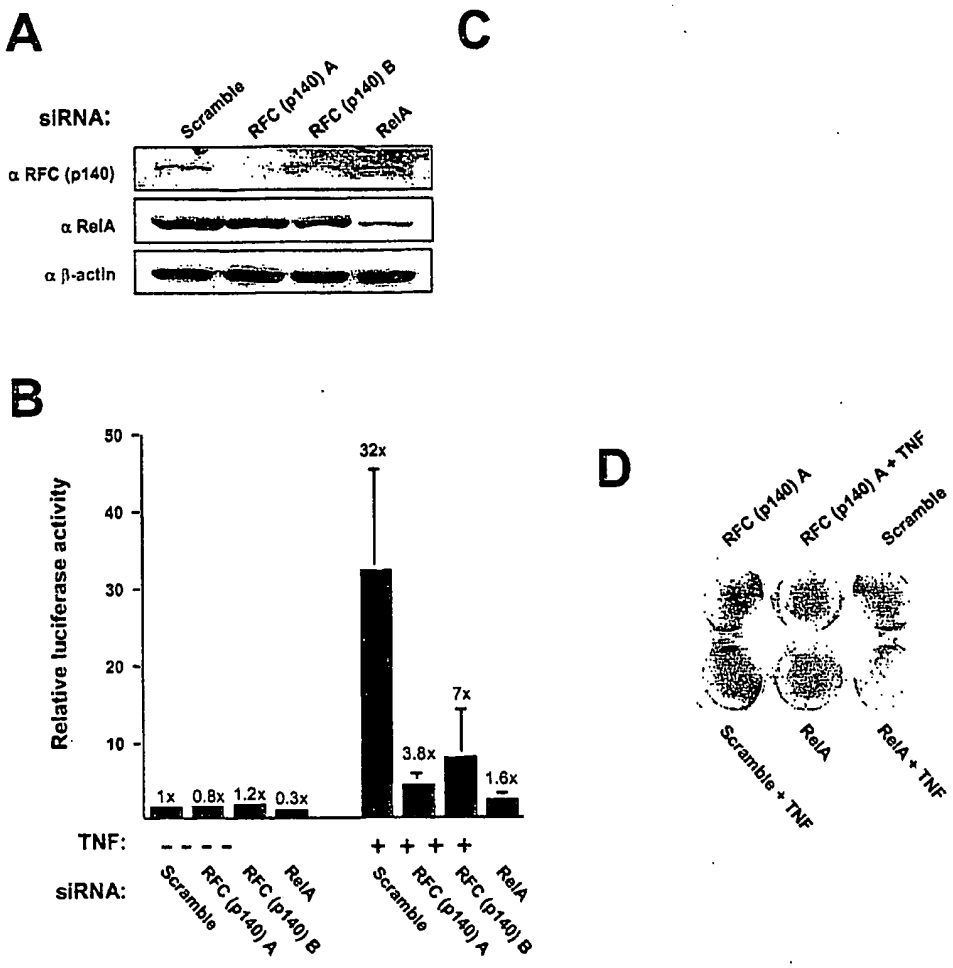

A
siRNA:
Scramble
RFC (p140) A
RFC (p140) B
RelA
α RFC (p140)
α RelA
α β-actin
C
B
Relative luciferase activity
50
40
30
20
10
32x
7x
3.8x
1.6x
1x
0.8x
1.2x
0.3x
TNF:
- - - -
+ + + +
siRNA:
Scramble
RFC (p140) A
RFC (p140) B
RelA
Scramble
RFC (p140) A
RFC (p140) B
RelA
D
RFC (p140) A
RFC (p140) A + TNF
Scramble
Scramble + TNF
RelA
RelA + TNF

Figure 6

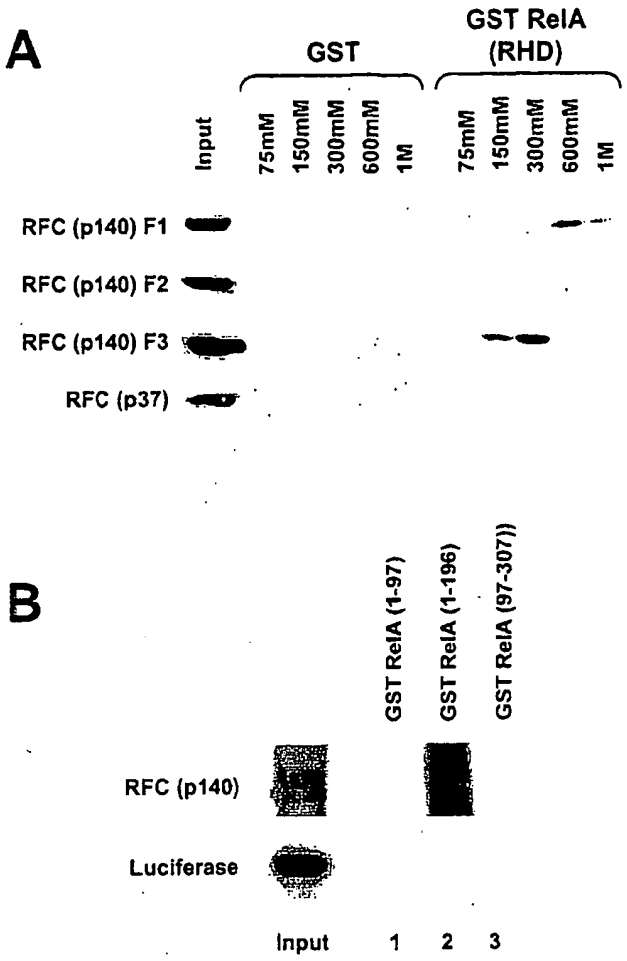

A
GST
GST RelA (RHD)
Input
75mM
150mM
300mM
600mM
1M
75mM
150mM
300mM
600mM
1M
RFC (p140) F1
RFC (p140) F2
RFC (p140) F3
RFC (p37)
B
GST RelA (1-97)
GST RelA (1-196)
GST RelA (97-307))
RFC (p140)
Luciferase
Input
1
2
3

Figure 7

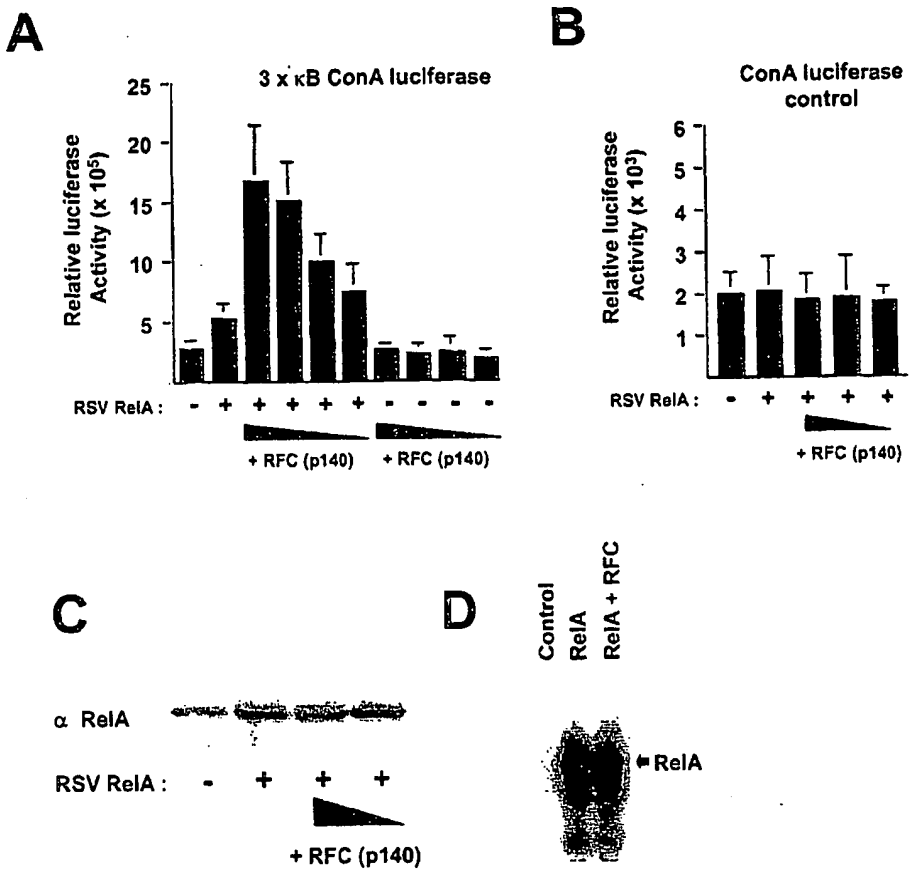

A
3 x κB ConA luciferase
Relative luciferase Activity (x 10⁵)
25
20
15
10
5
RSV RelA : - + + + + + - - - -
+ RFC (p140)
+ RFC (p140)
B
ConA luciferase control
Relative luciferase Activity (x 10³)
6
5
4
3
2
1
RSV RelA : - + + + +
+ RFC (p140)
C
α RelA
RSV RelA : - + + +
+ RFC (p140)
D
Control
RelA
RelA + RFC
RelA

Figure 8